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Lipophilic 9,10-dehydrofukinone action on pathogenic and non-pathogenic bacterial biofilms. Why is this main volatile metabolite in *Senecio*?

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Abstract

The effect of a natural sesquiterpene ketone, 9,10-dehydrofukinone (DHF), on pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains isolated from chronic infectious processes, was the focus of the present study. Lipophilic DHF produced important antibacterial synergistic effects in association with ciprofloxacin (CPX) against two biofilm-forming strains of *S. aureus* HT1 (FIC = 0.21) and *P. aeruginosa* HT5 (FIC = 0.05). Hence, this mixture constitutes an excellent strategy to combat these biofilm-producing bacteria that overexpress drug efflux pumps as a resistance mechanism. Additionally, a substantial rise in beneficial *Lactobacillus* biofilm biomass was determined as a very significant finding of this association. Particularly, a non-pathogenic biofilm increment of 119% was quantified when the mixture was added to a probiotic *L. acidophilus* ATCC SD-5212 culture. A surface activity enhanced in 71% with respect to untreated *L. acidophilus* culture was also generated by the DHF and CPX association, and therefore a glycoprotein synthesis induction mediated by the mixture is discussed. The results obtained could help in the development of new selective antibiotics. From an ecological standpoint, the present study strongly suggests that DHF is a polyfunctional organic molecule produced with a high yield in *Senecio punae* that exerts a positive impact on a non-pathogenic plant bacterium *L. plantarum* CE105.

Keywords:

Plant-derived sesquiterpene ketone

9,10-dehydrofukinone

Selective synergistic interactions

Pathogenic and non-pathogenic biofilm-producing bacteria

Surface activity

Introduction

Resistance to antibiotics is an urgent public health problem, as new resistance mechanisms are emerging and are spreading worldwide, interfering with the treatment of common infectious illnesses. A growing list of infectious illness is becoming recalcitrant to therapy and extremely difficult to eradicate with traditional antibiotics. Tackling antibiotic resistance is a high priority for the World Health Organization.[1] *Pseudomonas aeruginosa* (Priority 1) and *Staphylococcus aureus* (Priority 2) are the most important resistant bacteria in need of urgent new treatments since they are involved in chronic infectious processes.

Understanding the mechanisms involved in resistance to antibiotics is of importance both for the responsible use of antibiotics in practice, and for the development of new antibacterial drugs to circumvent resistance.[2]

From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic "attack": gene mutation, often associated with the mechanism of action of the compound, and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer.[3] Particularly, extrusion pumps provide an adaptation mechanism by which antibiotics are actively eliminated from bacterial cells and they are highly active in bacterial biofilms.[4]

Biofilms are dynamic habitats that constantly evolve in response to environmental fluctuations and thereby constitute remarkable survival strategies for microorganisms.[5] These are a serious global health concern due to their abilities to tolerate antibiotics, host defense systems and other external stresses; therefore it contributes to persistent chronic infections. A biofilm comprises of the crammed bacterial population by an extracellular matrix that possesses bacterial secreted polymers such as exopolysaccharides, extracellular DNA, proteins and amyloidogenic proteins.[6]

Reduced antibiotic susceptibility of bacteria in biofilms is understood to be due to a combination of poor antibiotic penetration, an altered microenvironment, adaptive responses, and the presence of bacterial persister cells.[7] Bacteria in biofilms show much greater resistance to antibiotics than their free-living counterparts. Hence, antibiotic-resistant bacteria are an obvious therapeutic objective, and the research of new antibiotic substances constitutes a potent tool for controlling the spread of resistant bacteria.[8][9]

Natural products are promising resources for non-conventional antibiotic discovery that can attenuate or prevent bacterial resistance or virulence factor expression. Previous studies carried out in our laboratory demonstrated that small molecules with different sesquiterpene skeletons and functionalizations, isolated from native plants, exerted important effects against pathogenic biofilms and other key virulence factors controlled by quorum sensing mechanisms.[10 – 14] Certain sesquiterpene lactones enhanced the antimicrobial activity of traditional antibiotics making them effective against pathogenic bacteria, but without harm to beneficial bacteria with probiotic potentialities.[15]

Lactobacilli, as bacterial symbionts that constitute the environmental and human microbiome, are considered as health-promoting bacteria due to their ability to produce various antimicrobial agents such as low molecular weight antimicrobials, bacteriocins, and adhesion inhibitors. Microbial surface active substances can interfere with pathogen adhesion on epithelial cells of intestinal and urogenital tracts, as well as on different surfaces and materials.[16] The antimicrobial and anti-adhesive properties of lactobacilli supernatants containing substances involved in quorum sensing and biofilm formation have been previously reported.[17][18]

Continuing with our investigations, herein we report the inhibitory activity of the main sesquiterpene ketone, 9,10-dehydrofukinone (DHF) (*Figure 1*) found in aerial

tissues of several species of *Senecio* (Asteraceae, Senecioneae), against biofilm-producing bacteria that overexpress drug efflux pumps, a fact determined by reactions with specific efflux pump inhibitors. Important synergistic interactions between DHF and ciprofloxacin on multidrug-resistant strains were also demonstrated, in agreement with our previous results.[19] The action of DHF on pathogenic and non-pathogenic bacteria was comparatively explored here. A significant surface activity was also noticed in the biofilm-forming *Lactobacillus* cultures in presence of DHF, and its probable ecological role and heath potential are hereby discussed for the first time.

Results and Discussion

Phytochemical analysis

The diethyl ether extract contained a main volatile sesquiterpene ketone, 4*β*,5*β*-eremophil-7(11)9-dien-8-one, also known as 9,10-dehydrofukinone, which was isolated as a pale yellow oil with a strong smell and unusual yield (2.1% related to the fresh plant material) by chromatographic techniques. IR, UV, EI-MS, ^{1}H and ^{13}C-NMR spectra were identical with previously reported data.[19 – 25]

The isolation of DHF as the main volatile natural product in *Senecio punae* (Asteraceae, Senecioneae) might have chemotaxonomic implications. Indeed, DHF, a volatile molecule with great versatility to carry out many different biological activities, has been found in aerial parts of *S. punae*,[19] *S. humillimus*,[22] *S. aureus*,[23] and *S. viridis* var. *viridis*.[25] The occurrence of this bioactive sesquiterpene ketone in aerial tissues of various species of *Senecio* strongly suggests its functional role against biotic stress.

Action of DHF and that of its mixture with ciprofloxacin on pathogenic and non-pathogenic bacteria

Pathogenic bacteria growth and biofilms

DHF effects and those of its mixture with ciprofloxacin (CPX) on the bacterial growth and biofilm biomass of *P. aeruginosa* and *S. aureus* are shown in *Figure 2*. While the DHF antibacterial activity was mild against *P. aeruginosa* (MIC = 6 mg ml^{-1}) and *S. aureus* (MIC = 1.5 mg ml^{-1}), the mixture of DHF (23 µg ml^{-1}) and CPX (0.25 µg ml^{-1}) exerted important synergistic interactions in the antibacterial activity against hospital isolates of *P. aeruginosa* HT5 (FIC = 0.05) and *S. aureus* HMR1 (FIC = 0.21).

S. aureus HT1 biofilm biomass after 24 h incubation in presence of DHF was significantly reduced by 68% at the MIC value ($P < 0.05$) and its association (25 µg ml^{-1}) with CPX (0.25 µg ml^{-1}) improved antibiofilm activity reaching 89% decrease of the biofilm biomass with respect to the control strain (without the addition of the mixture).

It is important to note that DHF produced significant growth and biofilm diminution on a methicillin resistant *S. aureus* strain at a lower concentration than MIC (67% and 11%, respectively at 0.75 mg ml^{-1}). In contrast, only the mixture exhibited a moderate reduction of the biofilm developed by *P. aeruginosa* HT5 (20%), as shown in *Figure 2*. Although DHF and its association with CPX were more active as antibacterial-antibiofilm agents against Gram (+) bacteria, this mixture showed a higher synergistic interaction on *P. aeruginosa* (FIC = 0.05). It is important to highlight that Gram negative pathogens generally exhibit a lower sensitivity to plant natural products than antibiotics as previously observed.[19][26][27]

Preliminary detection of efflux pumps in antibiotic resistant strains

Indole alkaloid reserpine, known efflux-pump inhibitor, associated with ciprofloxacin in lower concentrations than MIC, modified bacterial susceptibility to the antibiotic.

The results shown in *Table 1*, revealed a significant drop in the MIC values of CPX ranging from 1.25 μg ml^{-1} to 0.25 μg ml^{-1} for *S. aureus* HT1, and from 5 μg ml^{-1} to 0.25 μg ml^{-1} for *P. aeruginosa* wild-type strain HT5. Likewise, PA*β*N, known efflux-pump inhibitor in Gram negative bacteria[28], increased the antibiotic activity on *P. aeruginosa* HT5.

The bioassay performed with efflux pump inhibitors would prove the existence of efflux pumps as an antibiotic resistance mechanism in the selected bacteria. The results obtained were consistent with previous studies that reported that reserpine is a natural metabolite that blocks efflux pumps (NorA for fluoroquinolones) located in *S. aureus* membranes.[29] As has been well documented, plants are sources of potential resistance modifying agents and the involved compounds include different classes of natural products, such as: terpenes, flavones, flavonolignans, and porphyrins.[30 – 32] According to Gibbons,[33] common characteristics of all these agents appear to be the high degree of lipophilicity, a feature which is key for the interaction with membrane bound efflux proteins, and the ability to overcome membrane impermeability.

A striking fact is that reserpine also modified *P. aeruginosa* HT5 susceptibility to CPX (*Table 1*), but in this case by a strong biofilm decrease (67%, *Table 1*) that improved the antibiotic entry into the bacterial cell. This fact agrees with a recent publication about the inhibitory effects of reserpine on *P. aeruginosa* quorum sensing mediated virulence factors and biofilm formation.[34] Additionally, PA*β*N, known efflux-pump inhibitor in Gram negative bacteria[28] allows us to corroborate the efflux pump overexpressing phenotype in the clinical isolate of *P. aeruginosa* HT5.

According to Kvist and coauthors,[4] biofilm formation has a positive correlation with the drug efflux pump activity. Thus, in the *S. aureus* HT1 strain, the probable attenuation of the protein pump could reduce the developed biofilm in relation to the control (*Table 1*). DHF (theoretical partition coefficient o/w of 4.814, and log P o/w = 3.3135) shared a similar synergistic action with reserpine (*Figure 2*).

Transmission electron microscopic findings

Lipophilic DHF at non-inhibitory concentrations of 23 μg ml^{-1} exerts important alterations on *P. aeruginosa* HT5 cell wall and, in some cases, loss of wall structural integrity (*Figure 3*). The synergistic effects found between DHF and CPX would be brought about by the lipophilic natural product that causes the bacterial wall disruption, thus facilitating the passage and activity of the antibiotic. These findings provide more evidence that lipophilic compounds such as terpenes act as solvents causing a disruptive effect on the bacterial cell wall.[15][35]

S. aureus HT1 ultrastructure did not show changes due to the action of DHF. Bacteria in division with preserved edges were observed both in treated and control samples in MET microphotographs (*Figure 4*). Also, DHF did not exert an antiadherent effect after 1 h of treatment, as explained in the *Supplementary Information* (see *Figure S1*). Therefore, these images and the DHF behaviour suggest that this sesquiterpene ketone could act as an attenuator of efflux pumps on *S. aureus*. Indeed, the presence of ciprofloxacin (pH 6.72 in MH medium) was evidenced by an extracellular pH decrease from 7.09 to 6.75 triggered by antibiotic, after 24 hours of incubation at 37 °C. This difference provides evidence of the extracellular localization of ciprofloxacin and explains the lack of activity thereof (at intracellular level). In contrast, antibiotic at 0.25

182 μg ml^{-1} exerted a full inhibition in presence of DHF at a sub-inhibitory concentration
183 (23 μg ml^{-1}).

184 In light of the results obtained, the mode of action of DHF is substantially different
185 for each of these strains. In *P. aeruginosa* HT5, this compound produces an alteration of
186 the architecture of the cell wall, which would facilitate the diffusion of antibiotics into
187 the cell. While in *S. aureus* HT1 DHF in the synergistic association would attenuate
188 drug efflux pumps by increasing bacterial strain susceptibility to antibiotics, as do other
189 lipophilic natural products.[29– 33] Thus, this association is effective in both cases.

190 *Non-target bacterial strains*

191 *Growth and biofilms*

192 DHF at 23 μg ml^{-1} and its association with CPX at the inhibitory concentration
193 against the pathogenic strains did not exert antibacterial effects against probiotic and
194 indigenous strains isolated from different substrates of high-mountain biome (*L.*
195 *acidophilus* ATCC SD5212, *L. paracasei* CE75 and *L. plantarum* CE105, respectively).
196 Indeed, DHF at 23 μg ml^{-1} increased the growth from 13.31 ± 0.57 to 33.33 ± 2.76% for
197 all *Lactobacillus* strains assayed, while the addition of the mixture produced a
198 significant enhancement in biofilm biomass in all cultures ($P < 0.05$), as shown in *Table*
199 *2*.

200 The most important effects on biofilm formation were observed in *L. acidophilus*
201 ATCC SD-5212 with increments of 69 ± 2.37% for DHF at 23 μg ml^{-1} and 119 ± 1.53%
202 for the mixture compared with the untreated control. DHF with a hundredfold higher
203 concentration produced a steep increase of the bacterial biofilm (112 ± 1.00 and 112 ±
204 2.72% for *L. acidophilus* and *L. paracasei*, respectively), and only the growths of *L.*
205 *acidophilus* and *paracasei* were slightly diminished by 5 and 6% ($P < 0.05$),

206 respectively. Lipophilic compounds are believed to be interspersed in bacterial
207 membranes, altering their structure and properties. Bacteria then produce a hydrophilic
208 exopolysaccharide that constitutes a physicochemical barrier to the intercalation, as an
209 adaptation strategy to a hostile environment.[36] This phenomenon might be useful to
210 explain the stimulation of biofilm formation produced by the natural stressor DHF.

211 These results are consistent with those found in our earlier works.[15][19] This finding
212 is very important since the biofilm developed by beneficial *L. acidophilus* strains would
213 improve their tissue adhesion and their probiotic properties as previously published.[37 –
214 39]

215 *Significant enhancement in surface activity of the Lactobacillus cultures*

216 *Lactobacillus* supernatants from the treated cultures with different amounts of DHF
217 alone and mixed with CPX after 24 h of incubation generated higher halos in the oil
218 spreading assay than the untreated culture supernatant (12 – 71%), which suggests a
219 clear increase in surface activity, particularly in the association at lower concentrations
220 of DHF and CPX. All supernatants were more effective than tween 80 (*Figure 5*).
221 Nevertheless, DHF, DHF with CPX, and the solvent system of the samples previously
222 added to the culture medium did not exert any activity by themselves. Therefore, the
223 rise in *Lactobacillus* supernatant surface activity would be due to an increase in surface
224 active substances by an induction to the biosynthesis thereof in the bacterial cultures.

225 The surface activity makes *Lactobacillus* a promising source of anti-adhesive
226 biomaterials with antimicrobial potential. Our result is in agreement with a previous
227 investigation that reported on typical biosurfactants, named surlactin, that are
228 glycoproteins,[17][40 – 42] released by *Lactobacillus* species and have anti-adherent
229 activity.[43] Indeed, a preliminary characterization by TLC and UV[44][45] of the surface
230 active substances isolated from the *L. acidophilus* culture revealed the presence of

glycoproteins (data not shown). Nevertheless, further studies are necessary to identify these bacterial substances.

Conclusions

In conclusion, DHF in association with CPX represents an important strategy to combat biofilm forming pathogenic bacteria that overexpress drug efflux pumps as a resistance mechanism. This low-dose association of each substance did not inhibit the growth, and increased biofilm formation and anti-adherent activity of the beneficial *Lactobacillus* strains. This finding could help in the development of a new generation of antibiotics that are biofilm-promoters of the lactic acid bacteria present in the native microbiome. For this possible therapeutic application lipophilic DHF could be solubilized in polyethylene glycol (PEG). PEG is the most widely used polymer in the drug delivery field and classified as safe (GRAS) by the FDA.

From an ecological standpoint, our exhaustive bibliographical revision[19 – 25] strongly suggests that DHF is a key marker of aroma and bioactivity in the volatile profile, produced with a high yield by many aromatic *Senecio* species, especially *S. punae*. In addition, this sesquiterpene ketone showed a positive impact on known beneficial bacteria, such as *L. plantarum* CE105, and the probiotic bacterium *L. acidophilus* ATCC SD-5212, as demonstrated in the present work.

Experimental Section

For thin layer chromatography (TLC), pre-coated SiO_2 plates (*Merck*, Kieselgel 60 F) were employed. Spots on the plates were detected using Godin's reagent followed by heating at 120 °C. SiO_2 60 (*Merck*, 70 – 230 mesh) was used for column chromatography. UV spectra were recorded on a *Shimadzu UV/VIS 160 A*

spectrophotometer. FT-IR spectra were measured on a *FT-Perkin Elmer-1600* spectrophotometer. Optical rotations were measured on a *HORIBA SEPA-300* high-sensitive polarimeter with MeOH as a solvent. ^{1}H- and ^{13}C-NMR spectra were recorded on a *Bruker 200* (200 MHz) spectrometer; *d* in ppm relative to Me_4Si as internal standard, *J* in Hz. EI-MS was obtained on a *Thermo Electron Trace*^{TM} *GC Ultra* coupled to a *Thermo Electron Polaris Q* ion trap mass spectrometer with a 30 m x 0.25 mm id DB-5 MS column. The identification of DHF was based on computer matching with the *NIST08* GC/MS library (USA), and since DHF is not commercially available, DHF used as a standard was obtained by exhaustive purification from *S. punae* aerial parts as already described.[19]

Plant material

S. punae Cabrera is an endemic high mountain shrub that grows in the Puna semi-desert region in the North of Argentina (3000-4600 m above sea level) where was collected during the flowering stage. A voucher specimen was deposited at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina (LIL 609967).

Phytochemical study

Only a few grams of fresh aerial parts were processed and extracted according to *Rodriguez et al.*[19] The main volatile sesquiterpene ketone, known as 9,10-dehydrofukinone was extracted with diethyl ether and isolated with an unusual yield (2.1% related to the fresh plant material) by routine chromatographic fractionations. IR, UV, EI-MS, ^{1}H and ^{13}C-NMR spectra were identical with previously reported data.[20 – 25]

4*β*,5*β*-eremophil-7(11)9-dien-8-one (**DHF**). $[\alpha]_D$ = +131.97 (*c* 0.0167, MEOH). R_f = 0.5 (SiO_2, Hexane-AcOEt 95:5). T_R: 40.35 min (*DB-5*, 30 m, 0.25 mm, ramp temperature). IR ν_{max} film ($CHCl_3$): 3020, 2970, 2860, 1670, 1620, 1470, 1380 cm^{-1}.

279 UV/Vis λ_{max} (MeOH) nm (log ε): 274 (1.39 sh), 248 (2.26), 208 (1.08). ^{1}H-NMR
280 ($CDCl_3$): 0.93 (*s*, Me(15)); 0.97 (*s*, Me(14)); 1.84 (*s*, Me(12)); 2.09 (*s*, Me(13)); 2.87(*d*,
281 *J* = 13, H–C(6)); 5.74 (*br s*, C(9)).^{13}C -NMR ($CDCl_3$): 15.4 (C(15)); 15.9 (C(14)); 21.9
282 (C(12)); 22.5 (C(13)); 26.4 (C(2)); 30.4 (C(1)); 32.5 (C(3)); 40.9 (C(4)); 41.8 (C(5));
283 42.4 (C(6)); 126.0 (C(9)); 128.1(C(7)); 142.1(C(11)); 168.7 (C(10)); 192.3 (C(8)).
284 GC/MS (EI, 70 eV): 219 (100, M + H^+); 218 (30, M^+); 203 (14, M^+ − Me); 189 (5, 219
285 − 2 Me); 175 (9, M^+ − Me – CO); 161 (12, 189 – CO); 147 (21, 175 − CH_2=CH_2).

286 *Microbiological analysis*

287 *Pathogenic microorganisms*

288 *Pseudomonas aeruginosa* HT5, a strain resistant to several antibiotics (azithromycin 25
289 µg, aztreonam 30 µg, ceftazidime 30 µg, cefepime 30 µg, imipenem 10 µg, meropenem
290 10 µg, piperacillin-tazobactam 110 µg, gentamicin 30 µg, and ciprofloxacin 5 µg) was
291 used.[46] The strain was grown for 24 h at 37 °C in Luria Bertani (LB) medium. Besides,
292 *Staphylococcus aureus* HT1, a methicillin resistant strain (methicillin 30 µg, gentamicin
293 30 µg, and azithromycin 5 µg) was also used. [47] This strain was grown in Müller
294 Hinton (MH) medium for 24 h at 37 °C. Both biofilm-producing strains were isolated
295 from chronic infections (Hospital isolates).

296 *Non-pathogenic microorganisms*

297 *Lactobacillus paracasei* subsp. *paracasei* CE75 and *L. plantarum* CE105 were isolated
298 from regional cheeses and plants of high-mountain biome (respectively)[15][48], while *L.*
299 *acidophilus* La-14 was a collection strain (ATCC SD-5212). These strains were grown
300 in PTYg medium (15 g l^{-1} peptone, 10 g l^{-1} tryptone, 10 g l^{-1} yeast extract, and 5 g l^{-1}
301 glucose, pH 6.0) for 24 h at 37 °C.

Bacterial growth

An overnight culture (18 – 20 h) of the *P. aeruginosa* strain was diluted to reach an appropriate bacterial density (10^5 CFU ml^{-1}) in a final volume of 200 µl. In the same way, the overnight culture of the *S. aureus* strain was diluted in MH medium. A volume of 190 µl was placed in each well of a microtiter polystyrene plate. Solutions containing different DHF amounts were prepared separately in DMSO/distilled and sterilized water (50:50), and 10 µl of each one were pipetted into the wells individually to obtain final concentrations of 23 – 6000 µg ml^{-1}. After 24 h incubation at 37 °C, bacterial growth was measured at 600 nm (*P. aeruginosa*) and 560 nm (*S. aureus*), using a microtiter plate reader (*Power Wave XS2*, *Biotek*, VT, USA). A DMSO/water (50:50) solution added to the diluted culture was employed as growth control, and the antibiotic ciprofloxacin (CPX) was also incorporated into the bioassay, as a negative control. It was also associated with DHF to determine synergism. CPX acts on the bacterial DNA gyrase target and is a known biofilm inhibitor of *P. aeruginosa* strains.[12] Solubility controls for each substance and concentration were carried out in all assays. Absorbance values were subtracted from the average of the corresponding assay when there was turbidity. Synergistic effects of the DHF and CPX mixture against both strains were determined according to the following formula:

FIC index = FICA + FICB = [A] / MICA + [B] / MICB, where "FICA, FICB" is the fractional inhibitory concentration of drug A and B, respectively; "MICA, MICB" is the minimum inhibitory concentration of drug A and B, respectively, and "[A], [B]" is the concentration of drug A and B, respectively. FIC index by checkerboard method is interpreted as follows: ≤ 0.5 synergy; > 0.5 and ≤ 4 additivity and > 4 antagonism.[49]

Additionally, the effect of the mixture of DHF and CPX was proven on non-pathogenic bacteria.

Biofilm formation assay

The biofilm quantification was done using a method based on a crystal violet stain according to a protocol previously reported[50] with several modifications.[46] Ciprofloxacin, a known biofilm inhibitor, was incorporated into the bioassay.[51]

Effects of efflux pump inhibitors against pathogenic strains

Alkaloid reserpine, a known efflux-pump inhibitor of multidrug-resistant bacteria was used to verify if bacteria modified their susceptibility to ciprofloxacin[29], as well as phenylalanine-arginine *β*-naphthylamide (Pa*β*N). Hence, mixtures of ciprofloxacin at lower concentrations than MIC, and 20 μg ml^{-1} reserpine or 50 μg ml^{-1} Pa*β*N that do not inhibit bacterial growth but block efflux pumps were evaluated. The second-generation fluoroquinolone, ciprofloxacin, is the most often used antibiotic as the main reference substrate of NorA efflux pump assays.[9]

The results of this bioassay would prove the occurrence of efflux pumps as an antibiotic resistance mechanism in the selected bacteria. Complementarily, biofilm formation inhibition and its correlation with the attenuation of drug efflux pump activity were also investigated in this work.

Electron microscopic study

In order to assess the effect of DHF, a suspension of each bacterium in MH or LB broth was exposed to a sub-inhibitory concentration (23 μg ml^{-1}) of DHF for 24 h. A suspension of each microorganism was used as control. After exposure, aliquots of treatment and control suspensions were separated from the media and processed using routine techniques for transmission electron microscopy.

For histological studies of samples (T and C) were fixed in Karnovsky's solution (formaldehyde 2.66%, glutaraldehyde 1.66%, and sodium phosphate buffer 0.1 M pH 7.4). Post-fixation was carried out with a solution of sodium phosphate buffer/2%

352 osmium tetroxide (OsO_4) to improve contrast. Preparations were dehydrated with
353 increasing alcohol concentrations (30, 50, 70, 80, 85, 90 and 100%) and embedded in
354 epoxy resin SPURR. Ultra-thin sections were made with an ultramicrotome, mounted
355 on copper grids and contrasted with uranyl acetate and lead citrate. The samples were
356 observed with a *Zeiss EM109* (Carl Zeiss NTS GmbH, Oberkochen, Germany)
357 transmission electron microscope (CIME-CONICET, Argentina).

358 *Lactobacillus supernatant surface activity*

359 *Oil spreading assay*

360 The oil spreading assay is a rapid and highly sensitive method for surface-active
361 substance detection[18][52] and it is a good choice to explore the surface activity of
362 *Lactobacillus* supernatants (from control and treated cultures).

363 For the assay, 20 µl of mineral oil were placed on a crystalliser (180 mm in
364 diameter) with demineralized water (250 ml). Then, 10 µl of each free cell supernatant
365 were gently put on the centre of the oil film. The diameters of clear halos (mm)
366 visualized under visible light were measured in quintuplicate with respect to the control
367 supernatant. Tween 80 was employed as a reference standard (positive control). The
368 negative controls were the PTYg medium (without bacteria) and solutions of each
369 compound assayed.

370 *Statistical Analysis*

371 Differences between means were evaluated by analysis of variance (ANOVA). For
372 group comparison tests were used Tukey and subtle differences were used. In all
373 statistical analyses, P values > 0.05 were not considered significant. *Statistix 10* data
374 analysis software for researches (2013) was used.

375 **Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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Author Contribution Statement

María C. Verni: DHF identification and biological experiments. *José A. Garay*: DHF identification and biological experiments. *Lucía Mendoza*: Extraction and isolation of DHF. *Alicia Bardón*: Manuscript reviewing. *Susana Borkosky*: Supervision of DHF isolation and identification. *Mario E. Arena*: Manuscript reviewing and collaboration with the work. *Elena Cartagena*: Design and supervision of the work and manuscript writing.

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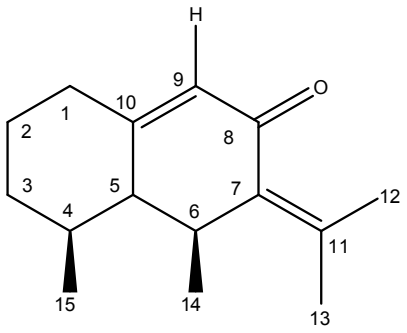

Figure 1. Eremophila-7(11) 9-dien-8-one (DHF) structure.

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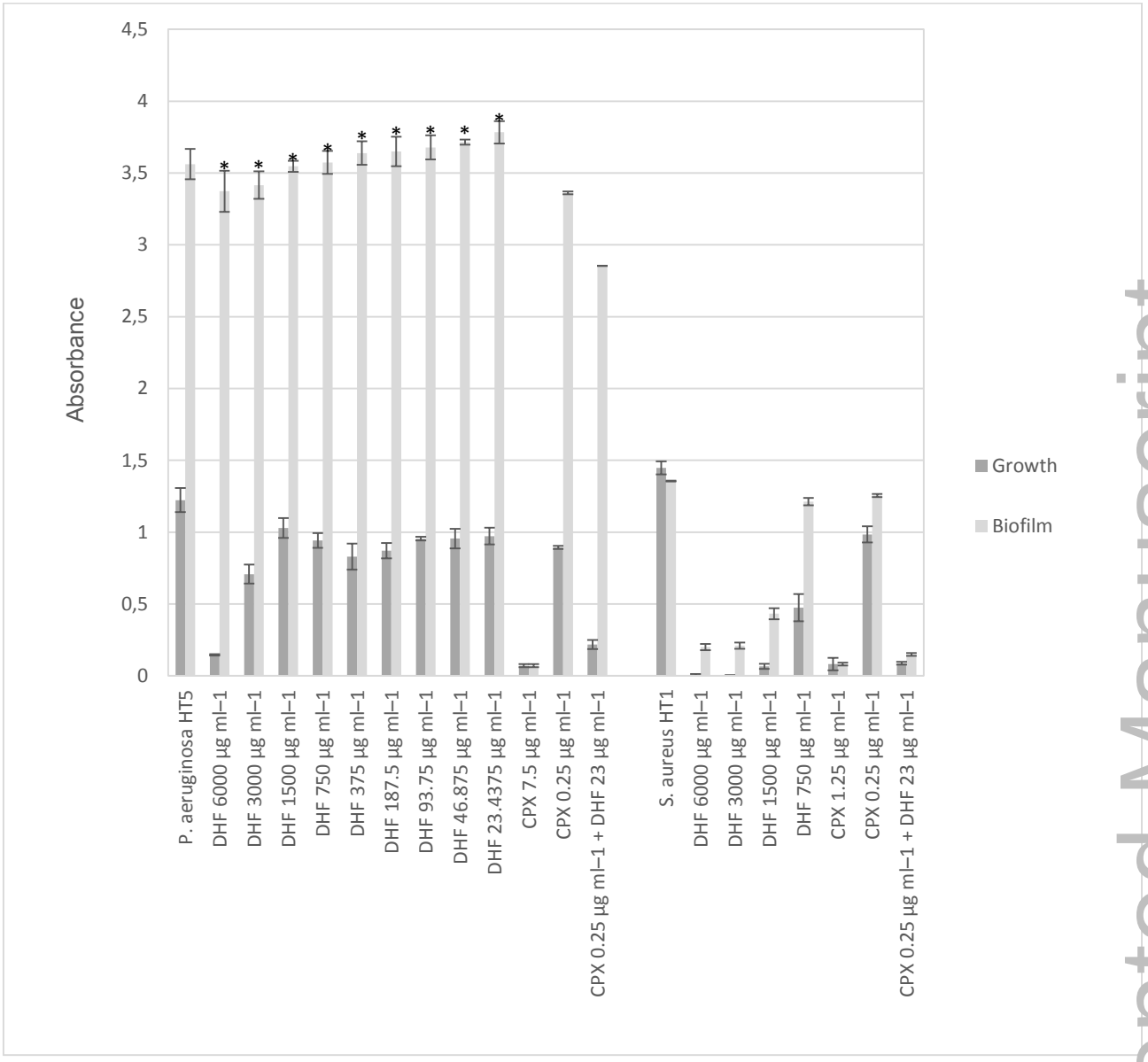

Figure 2. Effect of DHF and its mixture with CPX on growth and biofilms of *P. aeruginosa* and *S. aureus*. DHF: 9,10-dehydrofukinone. CPX: Ciprofloxacin. All experiments showed significant differences with respect to controls ($n = 8$, $P < 0.05$) except for bars with asterisks ($P > 0.05$).

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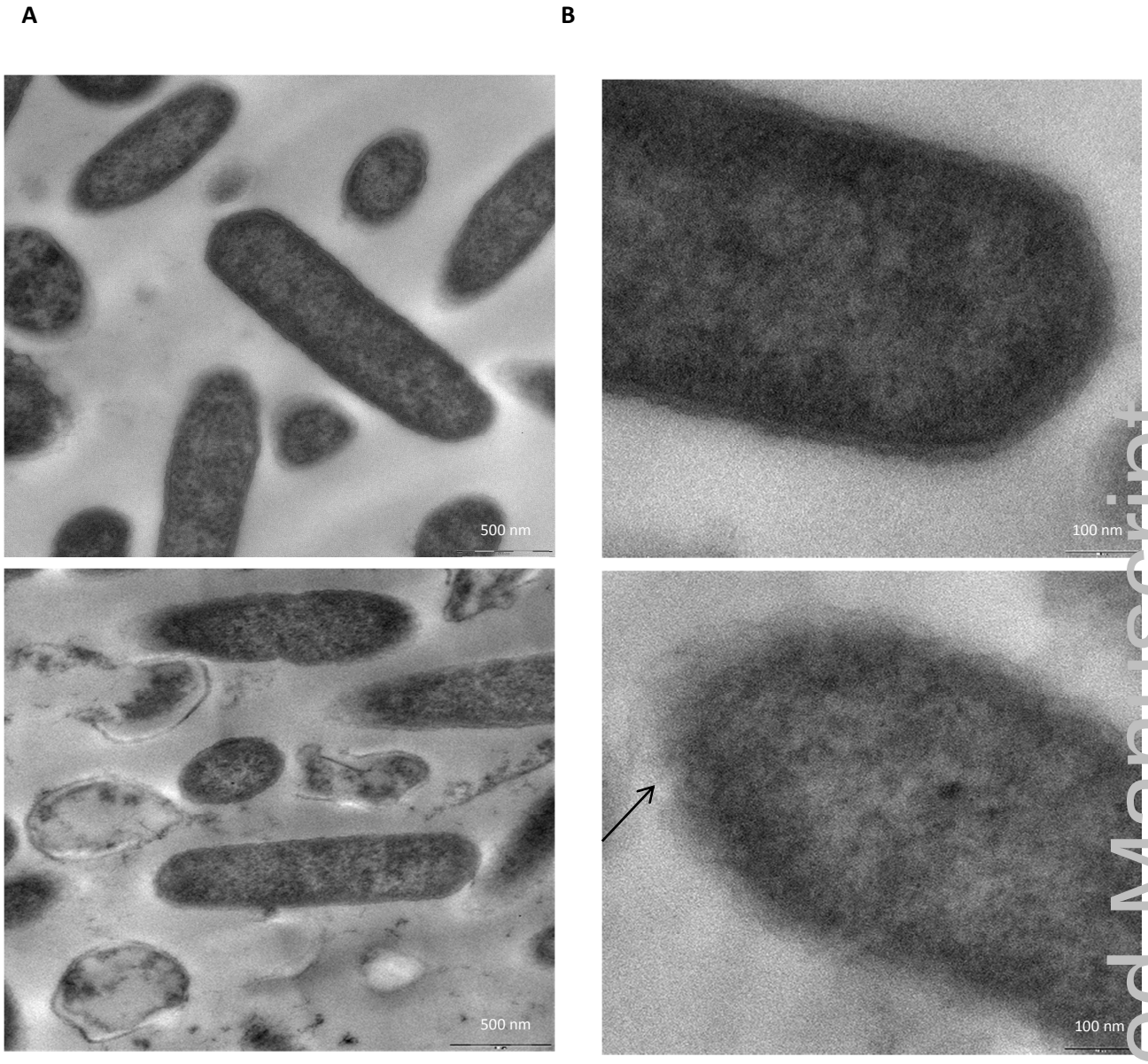

Figure 3. Transmission electron microscopy microphotographs of *P. aeruginosa*. Control (top of figure) and treated cells with a sub-inhibitory concentration (23 μg ml^{-1}) of DHF for 24 hours (bottom of figure) at a magnification of 10000x (*A*) and 40000x (*B*). Arrows indicate loss of wall structural integrity.

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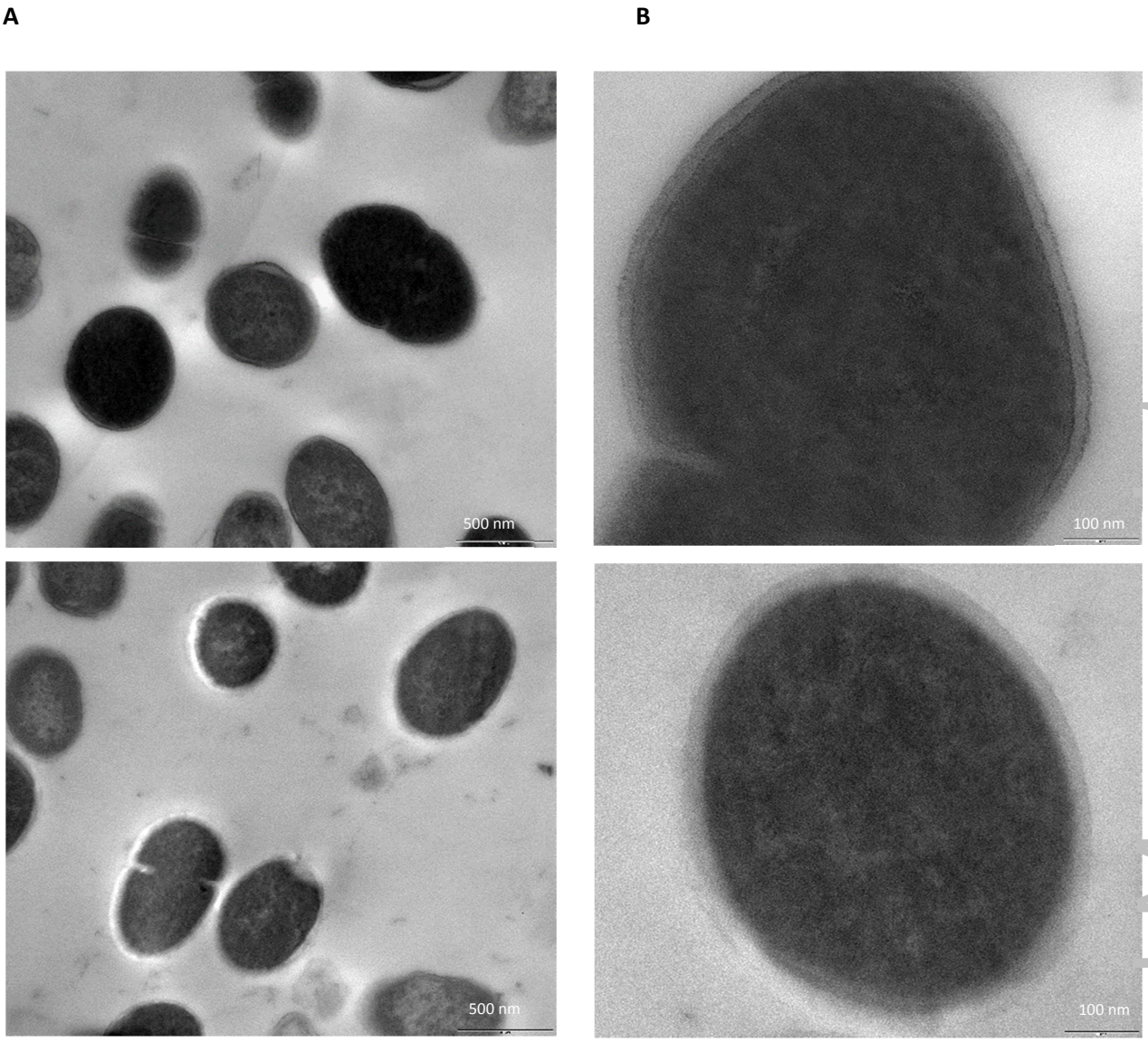

Figure 4. Transmission electron microscopy MET microphotographs of *S. aureus*. Control (top of figure) and treated cells with a sub-inhibitory concentration (23 μg ml^{-1}) of DHF for 24 hours (bottom of figure) at a magnification of 10000x (*A*) and 40000x (*B*).

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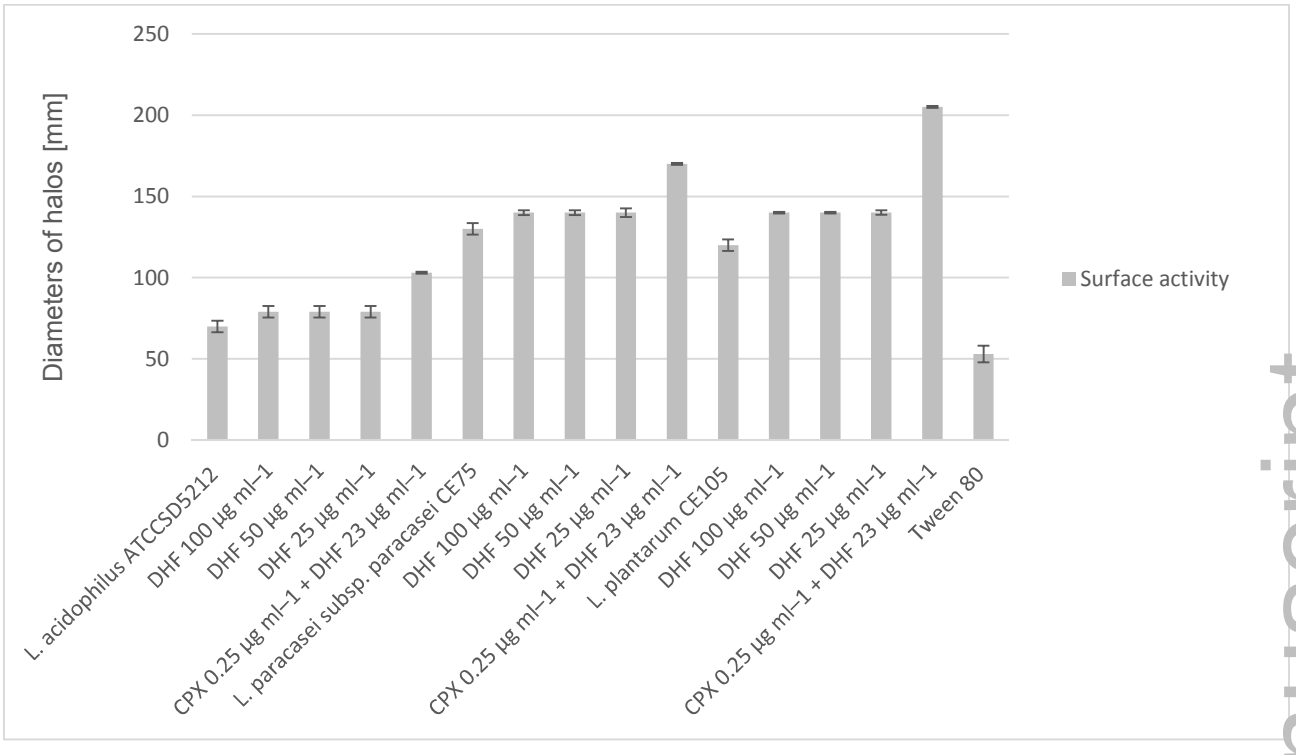

Figure 5. Effect of DHF and its mixture with CPX on surface activity of the *Lactobacillus* supernatants. DHF: 9,10-dehydrofukinone. CPX: Ciprofloxacin. All experiments showed significant differences with respect to controls ($P < 0.05$). DHF, DHF with CPX, and the solvent system of the samples previously added to the culture medium (without bacteria) did not exert any activity by themselves.

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Table 1. Effects of efflux pump inhibitors and their mixtures with CPX against two pathogenic strains isolated from hospital

Samples	**Bacterial strains**			
	***Pseudomonas aeruginosa* HT5**		***Staphylococcus aureus* HT1**	
	Growth	Biofilm	Growth	Biofilm
	[Means ± SD]			
Bacteria	0.903 ± 0.014 *a*	1.644 ± 0.083 *b*	1.532 ± 0.033 *a*	2.949 ± 0.177 *b*
CPX [MIC]	0.071 ± 0.010 *e*	0.091 ± 0.011 *e*	0.082 ± 0.044 *d*	0.092 ± 0.010 *e*
Reserpine [20 µg ml^{-1}]	0.814 ± 0.004 *b*	3.412 ± 0.047 *a*	1.449 ± 0.047 *a*	3.299 ± 0.129 *a*
Reserpine [20 µg ml^{-1}] + CPX [0.25 µg ml^{-1}]	0.204 ± 0.006 *d*	0.542 ± 0.012 *d*	0.269 ± 0.006 *c*	0.2893 ± 0.010 *c*
Pa*β*N [50 µg ml^{-1}]	0.876 ± 0.015 *a*	-	-	-
Pa*β*N [50 µg ml^{-1}] + CPX [0.25 µg ml^{-1}]	0.215 ± 0.005 *d*	-	-	-
CPX [0.25 µg ml^{-1}]	0.6593 ± 0.011 *c*	1.516 ± 0.010 *c*	1.042 ± 0.056 *b*	2.731 ± 0.010 *d*

CPX: Ciprofloxacin (MIC against *P. aeruginosa* HT5 = 7.5 µg ml^{-1}; MIC against *S. aureus* HT1 = 1.25 µg ml^{-1}). Pa*β*N: Phenylalanine-arginine *β*-naphthylamide at 50 µg ml^{-1}. (-): Not determined. Values within the same column with different superscripts are significantly different ($n = 8$, $P < 0.05$).

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Table 2. Effect of the DHF and CPX mixture on *Lactobacillus* cultures

Samples	**Bacterial strains**					
	***L. acidophilus* ATCC 521**		***L. paracasei* subsp. *paracasei* CE75**		***L. plantarum* CE105**	
	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm
	[Means ± SD]					
Bacteria	1.540 ± 0.018 *b*	0.924 ± 0.040 *d*	1.553 ± 0.024 *c*	1.353 ± 0.082 *d*	1.140 ± 0.029 *d*	1.781 ± 0.046 *c*
DHF [23 μg ml^{-1}]	1.745 ± 0.010 *a*	1.559 ± 0.037 *b*	1.760 ± 0.044 *a*	1.553 ± 0.062 *c*	1.520 ± 0.042 *b*	2.023 ± 0.065 *b*
DHF [230 μg ml^{-1}]	1.458 ± 0.019 *c*	1.959 ± 0.038 *a*	1.465 ± 0.017 *d*	2.868 ± 0.078 *a*	1.138 ± 0.032 *d*	1.798 ± 0.058 *c*
DHF [23 μg ml^{-1}] + CPX [0.25 μg ml^{-1}]	1.572 ± 0.028 *b*	2.021 ± 0.031 *a*	1.667 ± 0.015 *b*	1.976 ± 0.059 *b*	1.363 ± 0.041 *c*	2.502 ± 0.085 *a*
CPX [0.25 μg ml^{-1}]	1.563 ± 0.015 *b*	1.063 ± 0.027 *c*	1.556 ± 0.030 *c*	1.555 ± 0.059 *c*	1.674 ± 0.005 *a*	1.937 ± 0.060 *d*

DHF: 9,10-dehydrofukinone. CPX: Ciprofloxacin. (-): Not determined. Values within the same column with different letter are significantly different ($n = 8$, $P < 0.05$).